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Retroviral expression vectors on the basis of HERV LTR sequences

The present invention relates to retroviral expression vectors bearing promoters which may be cell-specifically controlled. The vectors are useful for example for the cell-specific expression of genes of therapeutic value in the context of a gene therapy.

Retroviruses are RNA viruses wherein the viral genes are encoded by a single-stranded RNA molecule. Following entry of the viruses into the cell, the viral RNA is converted into a double-stranded DNA molecule by means of reverse transcription. The DNA enters the nucleus and integrates into the cellular chromosome. The integrated form of viral DNA, the so-called provirus, represents the template for the expression of viral genes.

Integration of the viral genome into the cellular chromosome is an obligatory step of viral replication and is mediated by virus-encoded enzymes. With few exceptions it appears that the viability of the infected cell is not or almost not affected by the presence of the retroviral genome in the cell, the expression of its genes and the formation of viral particles.

Retroviral gene transfer is used for the introduction of functional genes, in particular genes of therapeutic value, into cells without affecting the ability of the host cell to proliferate. Due to their mode of replication retroviruses are suitable for such gene transfer. In the most simple

embodiment, at least a portion of the viral genes is replaced by a gene of interest and, by using the efficient viral infection process, this gene of interest is transferred into the target cell.

Retroviral vectors are suitable for gene therapy because the infection by retroviruses occurs with high efficiency and the retroviral vectors may be modified to incorporate heterologous DNA and may stably integrate into the genome of the host cell. A plurality of retroviral vectors has been developed in recent years, and by way of example reference is made herein to the reviews by Günzburg et al. (1996) and Robbins et al. (1998).

A possible preferred embodiment for retroviral vectors are so-called ProCon vectors which have been described for the first time in WO 96/07748. For the disclosure reference is made to this document in its entirety.

ProCon vectors have heterologous promoter elements and optionally other regulatory elements in their 3' LTR which following infection are duplicated and translocated to the 5' LTR in the target cell and which are capable of controlling the expression of marker genes or therapeutic genes. These heterologous genes are not directly linked to the promoter but are inserted inside the vector.

ProCon vectors comprise an 5' LTR portion having the structure U3, R, U5, and at least one coding and/or non-coding sequence as well as a 3' LTR region comprising a U3 portion which is completely or partially deleted wherein the deleted U3 portion has been replaced by a polylinker sequence followed by the R and U5 portions.

Propagation of these vectors is performed by means of a helper cell line producing high amounts of viral proteins which are no longer synthesized by the expression vector itself. However, the helper cell line is no longer capable of producing a replication-competent virus. This cell line is also referred to as packaging cell line and comprises a cell line transfected with at least a second plasmid carrying the genes which enable the packaging of the modified retroviral vector. In this respect, reference is made to WO92/10564 which is incorporated herein by reference in its entirety.

The DNA encoding the modified retrovirus (expression vector) is transfected into the packaging cell line. Under these conditions the modified retroviral genome comprising the inserted therapeutic genes or marker genes, respectively, is transcribed and packaged into retroviral particles (recombinant viral particles). Then, this recombinant virus is used for the infection of target cells; the genome of the modified retrovirus, i.e. the expression vector, is integrated into the target cell genome, wherein this integration occurs together with the marker genes or therapeutic genes, respectively. A cell infected with the recombinant viral particles generated in this manner is unable to produce new vector virus since no other viral proteins are present in these cells. The DNA of the expression vector containing the genes of therapeutic value or marker genes, respectively, which has been integrated into the host cell is present in the cellular DNA in an integrated form and may subsequently be expressed in the cell.

Preferably, genes of therapeutic value for which an expression is achieved by means of such retroviral expression

vectors are to be expressed in a cell- and tissue-specific manner. For this purpose, cell-specific regulatory sequences are introduced into the LTR sequence of the expression vector. For example, these cell-specific regulatory sequences comprise cell-specifically controllable promoter regions, cell-specific enhancer sequences as well as binding sites for transcription factors. The promoters are localized in the U3 portion of the LTR.

Presently, cellular promoter sequences or promoter sequences of exogenous retroviruses are inserted into retroviral vectors.

Cellular promoters often require additional signal structures which may be present at a great distance upstream or downstream of the promoter. Therefore, it has always been found difficult to isolate strong, tissue-specific, cellular promoter sequences and to clone them into retroviral vectors. Promoters of exogenous retroviruses bear the advantage that they contain, within the retroviral LTR, all the necessary regulatory elements in a confined region and, therefore, may be transcribed essentially independent of neighboring DNA sequences at the site of integration. However, a severe disadvantage is that although they are strong, they are not tissue-specific and generally are expressed with equal strength in all cell types.

Therefore, it is an object of the present invention to provide novel retroviral expression vectors which utilize the benefits of retroviral promoters to concentrate all signal structures required for transcription in a confined region within the U3 and R regions, but simultaneously avoid the disadvantages associated therewith.

According to the present invention, this object has been achieved by inserting into retroviral expression vectors a cell-specifically controllable promoter portion derived from a human endogenous retroviral DNA nucleotide sequence (HERV). These promoter sequences of human endogenous retroviral viruses are already present in the host cell, and it has been found according to the invention that they are excellently suitable for regulation of the cell-specific expression of marker genes and genes of therapeutic value.

Endogenous retroviruses (ERV) may be found in the genome of all cells in an organism. They are transferred vertically via the germ line and may be reactivated by conditions caused by the environment. About 2% of the human genome consist of endogenous retroviruses and retroviral sequences, solitary HERV LTRs being present in an amount of 20,000-40,000 copies per genome (Tab. 1) (Leib-Mösch et al., 1993; Wilkinson et al., 1994; Patience et al., 1997).

Since HERV sequences have been integrated into the primate genome already 30-40 millions of years ago, it may be assumed that in the course of evolution most of the pathogenic sequences were eliminated from the provirus by mutations and rearrangements or have been modified, respectively, to be no longer disadvantageous for the organism. Compared to vectors derived from animal viruses, retroviral vectors constructed from such sequences have the advantage that no new viral sequences must be introduced into the genome. In addition, also by recombination with HERV sequences already present in the genome no novel retroviruses may arise as it might be the case if retroviruses of other species were used as vectors. For this reason, the use of these sequences in the

construction of retroviral vectors can minimize the safety risk. Furthermore, homologous regions contained in the genome may be utilized for a tissue-specific integration of the retroviral vectors into specific sites of a chromosome.

In the course of evolution, HERV elements have adopted a number of cellular functions. For example, promoter and enhancer elements of HERV LTRs are used for the transcriptional control of cellular genes (Kato et al., Feuchter-Murthy et al., 1993; Di Christofano et al., 1995). One example for the use of LTR regulatory elements for a tissue-specific expression of a cellular gene is the human amylase gene. This gene is controlled by the LTR of an HERV-E element and in this manner is restricted specifically to be only expressed in saliva glands (Ting et al., 1992). Moreover, Schulte and co-workers (1996) have shown that the insertion of an endogenous retrovirus into the 5' untranslated region of the pleiotrophin gene is responsible for the throphoblast-specific activity thereof (Schulte et al., 1996). In other instances, polyA signals of HERV LTRs may also serve to polyadenylate cellular transcripts (Mager, 1989; Goodchild et al., 1992).

Since retroviruses must maintain their transcriptional activity most independently of the surrounding regions of their site of integration, the main advantage of the use of retroviral promoters resides in the fact that all signal structures required for transcription are localized in a confined region within the U3 region and the R region of the LTR, as described above. Because these HERV promoters have persisted in the primate genome since millions of years they have adapted during evolution to be active in a cell type-

specific manner similar to cellular promoters and thus combine the advantages of cellular and retroviral promoters.

HERVs are transcribed starting from a classical RNA polymerase II promoter (Wilkinson et al., 1994). This promoter is localized within the LTR region. Therefore, the HERV transcript comprises no complete copy of the provirus. To compensate for the loss of transcription control elements, these elements have developed the mechanism of reverse transcription by which the lost sequences at both ends of the elements are regenerated from which in turn the LTRs are regenerated. Besides promoter sequences the HERV LTRs also contain a plurality of different binding sites for transcription factors (Seifarth et al., 1998) responsible for the tissue-specificity of expression.

Although there is a certain structural similarity between HERVs and exogenous animal retroviruses, such as MLV or MMTV, HERV sequences and in particular HERV promoters have never been considered as possible candidates for the development of retroviral expression vectors. In contrast, up to now they have only been considered as disruptive factors in view of gene therapy (Patience et al., 1997). Because of sequence homologies, there has been concern that they might interfere with the therapeutic vector by recombination in the target cell. Although it was impossible up to now to confirm these concerns by experimentation, however, a problem arose in the development of very efficient human packaging cell lines that co-packaging and inadvertent transfer of potentially infectious HERV sequences occurred. Therefore, a detailed study of the possible packaging of expressed HERV sequences into virions based on MLV has been conducted. Patience et al. (1998) identified mRNA transcripts of several different HERV

families, such as HERV-K and HERV-H, in human packaging cell lines. Even by using a highly sensitive RT-PCR test, however, none of these sequences could be detected in the MLV vector particles released by the cells.

According to these findings, a packaging and transfer of HERV sequences and therefore, eventually, also of HERV-based vectors in MLV packaging systems seemed to be out of question. Although HERV genes have a sequence homology of 50-65% with respect to MLV genes, particularly the regions which are essential for packaging and infection, and in particular the packaging signal localized between the 5' LTR and the gag region as well as the LTR itself have no detectable sequence homology with respect to the corresponding MLV sequences. Since up to now there are no cell lines known which produce HERV particles in sufficient amounts, however, presently, efficient HERV packaging systems are also not conceivable.

Thus, the present invention solves the problem of retroviral expression vectors controlling the cell- and tissue-specific expression of foreign genes (gene of interest) by providing expression vectors containing, in functional assembly, at least the following elements:

a) DNA sequences for packaging of the vector RNA and for the cell-specific expression of proteins or peptides encoded by heterologous DNA nucleotide sequences;
b) one or more heterologous DNA nucleotide sequences (transcription unit) encoding a protein or peptide;
wherein the DNA sequences for cell-specific expression are characterized by comprising a cell-specifically controllable promoter region derived from a human endogenous retroviral virus, in particular from the LTR sequence of said virus.

The promoter region of the HERV sequence may comprise the whole LTR region of the HERV. However, in another embodiment of the present invention, the promoter region only comprises the U3 region or the R-U3 region of the HERV LTR. In another preferred embodiment of the present invention, besides these regions the promoter region also comprises the untranslated region between the 5' LTR and the gag genes. It has been found according to the present invention that this region also contains sequences which control the cell-specific expression of proteins or peptides, respectively, i.e. which at least contribute to said cell-specific expression.

The promoters are partial regions of DNA required for the start of transcription of the corresponding structural genes. The promoter includes the transcription start site, the recognition and binding site for RNA polymerase. The promoter may also comprise other sequences to which regulatory proteins may bind and which thereby specifically control the initiation of transcription. Examples of such proteins are transcription factors and repressors. Examples of said regulatory elements of the transcription activity are the CAAT box, GC box and TATA box. The promoters are recognized by a type II polymerase.

The promoter regions for the cell-specific expression of foreign proteins from HERVs may optionally be combined with other sequences derived from exogenous retroviruses which promote cell-specific expression. In addition, there is considered a combination with regulatory sequences from cellular genes to support cell-specific expression.

Furthermore, the retroviral expression vector according to the present invention at least contains DNA sequences for packaging of the vector by means of a packaging helper cell line. The DNA sequences for packaging are localized between the 5' LTR and the gag gene. Such packaging signals are present in any retroviral vector and therefore known to the skilled artisan. Examples of packaging signals are listed in Mann et al., 1985, and Rein, 1994, as well as the literature cited therein. This literature is incorporated by reference in its entirety.

The retroviral expression vector according to the present invention contains one or more transcriptional units encoding an amino acid sequence. The amino acid sequence refers to a protein or peptide. Any sequence encoding a protein or peptide of interest may be inserted into the expression vector. For example, such proteins or peptides may be encoded by marker genes, genes of therapeutic value, genes with antiviral function, anti-tumor genes and/or cytokin genes. This list could be continued to any number. The genes which can be introduced into the retroviral expression vector are known to those skilled in the art. The type of genes inserted depends on the intended use of the vector according to the present invention.

For example, the vectors according to the present invention may be employed for gene therapy to transfer heterologous DNA into target cells in order to render diseases accessible to a specific therapy. The vector DNA is introduced into the selected target cell so that the heterologous DNA is expressed in the target cell and the product encoded by the DNA is produced. This includes particularly such genes for the expression of proteins which are not produced or not

produced any longer or not produced in sufficient amounts by the target cell so that a disease condition develops. The invention not only comprises such proteins or peptides, respectively, which occur naturally but also those which have been modified in a manner to achieve a desired effect, for example a higher enzyme activity, blocking of a binding site for viruses, destruction of tumor cells by suicide genes, etc.

Generally, the DNA nucleotide sequences encoding a protein or a peptide is heterologous DNA encoding RNA and proteins which the cell in which the proteins or peptides, respectively, are expressed usually does not produce in vivo. It may also be referred to as foreign DNA. This includes any protein, such as enzymes, hormones, and antibodies. Therefore, the retroviral expression vectors provided by the present invention are designed to express proteins of interest in human cells.

The promoter regions employed according to the present invention are selected from HERV sequences derived from the HERV families known. Examples of these are HERV-K, HERV-H, HERV-E, HERV-L, HERV-T, HERV-R, HERV-I, HERV-P, ERV9, HERV-W.

It should be understood that it is also possible to screen other, presently unknown HERV families in order to find promoter sequences which are presently unknown and which regulate the cell-specific expression.

Preferred LTR sequences from HERVs according to the present invention which may be employed for the tissue-specific expression of proteins and peptides are disclosed in the annex. They may be introduced into retroviral expression

vectors to achieve the object according to the present invention. It should be understood, however, that by means of methods known per se it is also possible to select only portions of these LTRs to keep the sequences inserted into the vector as small as possible. Useful fragments may be selected using various deletion mutants. Furthermore, also other variations of these LTR sequences are possible, e.g. point mutations, insertions, additions, substitution of several nucleotides, etc. in order to increase the efficiency of the tissue-specific expression and to adapt it to the desired function.

In a preferred embodiment according to the present invention the ProCon vectors described at the beginning are employed. Such ProCon vector comprise a 5' LTR region having the structure U3-R-U5, one or more sequences encoding a protein or peptide and optionally non-coding sequences as well as a 3' LTR portion comprising a partially or completely deleted U3 region wherein the deleted U3 portion at least comprises the HERV LTR sequences employed according to the present invention, followed by an R-U5 region. Further details are described for example in WO96/07748 and WO96/28564. These documents are included herein by reference in their entirety.

According to the present invention a strategy has been developed to track down promoter sequences having a cell-specific function. This strategy is described in more detail in the following description. It has to be understood that principally also other methods for the finding of HERV LTR sequences which act in a cell-specific manner may be considered and used. Thus, the present invention is not limited to the following examples.

The retroviral expression vectors according to the present invention are packaging deficient, i.e. are unable to produce viral particles without assistance by a packaging helper cell line. Therefore, the present invention also comprises a retroviral vector system containing a retroviral expression vector as described in the present invention and a packaging cell line which contains at least a retroviral or recombinant retroviral construct encoding the packaging proteins of the retroviral expression vector. Such packaging cell lines are known per se and have been described. By way of example, reference is made herein to the murine packaging cell line PA317 (Saller et al., 1998).

In the following, the invention will be described in general, followed by a description with respect to Examples.

According to the present invention, the applicability of human endogenous retroviruses for the development of tissue-specific vectors for gene therapy has been investigated. For this purpose, first the tissue-specificity of HERV pol transcription has been examined in different cell lines, such as T cells, keratinocytes and breast cancer cells using a "reverse dot blot" procedure. In this test, the expression pattern of the various HERV families was found to be generally cell type dependent. To isolate HERV LTRs with transcriptional activity from different cell lines and tissues, primers were developed which could be used for the specific amplification of the U3/R regions from mRNA preparations. The isolated LTR sequences as well as individual members of already known LTRs were inserted into expression vectors. Following transient transfection of the reporter plasmids, the activity of the LTR promoters was tested in the different cell lines via the luciferase

activity or eGFP fluorescence, respectively. The promoter activity of individual HERV LTRs was found to vary clearly dependent on the cell line tested. The promoter region of a HERV-H LTR isolated from astrocytes and liver cells which was found to be especially active in lung fibroblast cells (LC5) in several tests was inserted into two retroviral promoter conversion vectors (pLESN and pLX), tested in packaging cell lines, the packaging efficiency was evaluated, and after infection of the target cell was tested for the occurrence of a promoter conversion. FACS analyses were performed to detect the transcriptional activity in the target cells.

Thus, a method has been described by which HERV promoter sequences (U3/R region) mediating a tissue-specific expression may be identified and isolated. Subsequently, the tissue-specificity and promoter activity of these sequences was tested in a transient transfection assay in various human cell lines. Eventually, suitable sequences were chosen, cloned into a promoter conversion vector (ProCon vector) whereby their usefulness for the construction of tissue-specific vectors for gene therapy was examined. The preparation of the retroviral expression vectors according to the present invention is carried out using recombinant techniques known per se. Such techniques are for example described in Sambrook et al., 1989, and Perbal, 1984. For the construction of the ProCon vectors see WO 96/07748 already mentioned at the beginning an the related literature.

3. Results

3.1 Analysis of HERV transcription in different cell types

To investigate HERV transcription in different cells a method (reverse dot blot hybridization) was employed in the first step which had been originally developed for the detection of HERV expression in peripheral blood mononucleated cells (Herrmann and Kalden, 1994). For this method, cloned and characterized HERV *pol* gene fragments from human genomic DNA were immobilized on a membrane and hybridized with radiolabeled HERV *pol* gene probes. The probes were amplified from mRNA of different cells using RT PCR and degenerated oligonucleotides homologous to a highly conserved region of retroviral *pol* genes (Shih et al., 1989; Donehower et al., 1990). Using this method we obtained a characteristic hybridization pattern with every cell line examined so far which was the first result to indicate a tissue-specific expression of HERV elements.

3.2 Isolation of LTR U3 regions of expressed HERVs

The tissue-specific expression of a retrovirus is primarily defined by its U3 region. In this region, all regulatory sequences are localized, such as promoter, enhancer, and binding sites for various cellular transcription factors. For this reason, primers were developed which could be used for the specific isolation by means of RT PCR of these HERV sequences from the mRNA of different cell lines (Tab. 2; Fig. 1). In this manner, about 30 different HERV LTRs were cloned. In part, these sequences were tested in a reporter plasmid for their promoter activity and tissue-specificity.

In a first approach, for the PCR a polydT primer was combined with a primer complementary to the polypurine stretch (PPT) of retroviral RNA (Fig. 1). The PPT stretch is a conserved portion in the non-translated region between the *env* gene and

the U3 region of the 3' LTR. During reverse transcription of the retrovirus, the PPT region is used as a primer binding site for plus strand synthesis (Sorge and Hughes, 1982).

By means of data base analyses, the PPT sequences of different HERV families were identified and classified into different groups by comparing their homology. From the consensus sequences of individual groups oligonucleotides were synthesized as primers for RT PCR. The mRNA was prepared from different cell lines: epithelial cells (HeLa, HaCaT), fibroblast cells (LC5), T cells (H9, HUT78), lymphoblasts (CML), glioma cells (85HG66, U373), pancreatic cells (MiaPaCa2, Panc1), liver cells (Chang Liver), and breast cancer cell lines (T47-D, MCF7). Moreover, cDNA libraries (Clontech) of various human tissues (brain, heart, liver, kidney, lung, pancreas, placenta, skeletal muscle) were also employed in the RT PCR.

Subsequently, the fragments obtained were cloned, sequenced, and analyzed by means of data base comparison. Among the PCR fragments obtained with PPT and polydT primers two LTRs were assigned to the families of HERV-H and HERV-K due to a comparison of homologies. By using polydT primers in these PCR samples, numerous sequences were amplified which did not reveal any homologies to known retroviral LTRs and moreover did not contain any promoter structural elements. For this reason, other sequences were selected for primer synthesis from conserved regions of the U3 region and from the R region of the HERV-K and HERV-H families (Mold et al., 1997) (Fig. 1, Tab. 1). The resulting PCR products were separated on an agarose gel, followed by transfer to nitrocellulose filters and hybridization with probes prepared from the LTR regions of different HERV LTRs (HERV-K-pl167, HERV-H-H6, HERV-E,

HERV-L). Afterwards, the hybridizing fragments were cloned into a vector (pZERO, Invitrogen) and sequenced. Using this method, several HERV LTRs could be isolated which are listed in Table 3.

The HERV-K LTRs isolated from human brain and heart tissue as well as from T47-D cells show very strong sequence homologies to the 3' LTR of HERV-K10. In contrast, the HERV-H LTRs exhibited much higher sequence variations. HERV-H31, HERV-H3, HERV-HCM1, HERV-HCM4, HERV-HMP23 are homologous to the HERV-H-H6 LTR isolated by Mager et al., the other HERV-H sequences show homologies to the HERV-H LTRs from vervet monkey, marmoset and man isolated by Anderssen et al. (1997). The HERV-W LTRs isolated from T47-D cells are related to the LTR of clone CL6 (Komurian-Pradel, 1989).

3.3 Analysis of the expression of HERV promoters in a transient luciferase assay

For an analysis of the promoter activity and tissue-specificity of the isolated HERV LTRs, these were first cloned into a luciferase reporter plasmid (pBL, Butz, K., DKFZ, Heidelberg). This vector contains the luciferase gene of *Photinus pyralis* fused to the SV40 polyA signal of pBLCAT2 (Hoppe-Seyler et al., 1991).

The individual vector constructs were transiently transfected into different cell lines. After 48 h, the luciferase activity from the cell lysate was measured using the luciferase assay kit of Promega company and was determined as the relative luciferase activity after standardization for ß-galactosidase activity or *Renilla* luciferase activity, respectively. The LTR promoter activities were determined in

epithelial cells (HeLa, HaCaT), fibroblast cells (LC5), T cells (H9, HUT78), glioma cells (85HG66, U373), liver cells (Chang Liver), pancreatic cells (MiaPaCa2, Panc1), and breast cancer cell lines (T47-D, MCF7).

The results are presented in Figures 2a-2f. According to these results, among all endogenous LTRs tested the HERV-H-H6 LTR has the strongest promoter. The HERV-K LTR from placenta is particularly active in HeLa cells. In all other cell lines, this LTR exhibits only a very weak activity. Also in HeLa cells, HERV-K-T47-D showed a strong activity, this LTR was also active in HaCaT cells and pancreatic cells. The HERV-L LTR has a strong promoter activity in liver cells and a weak activity in T cells and pancreatic cells. The HERV-T-S71A and HERV-E LTRs were active in none of the cell lines tested. Also, no activity at all of a HERV LTR could be observed up to now in CML cells.

Almost all of the cloned HERV-H LTRs (HERV-H1, HERV-H8, HERV-H13, HERV-H19, HERV-H H6, Tab. 3) were active in 85HG66 cells while HERV-H1 and HERV-H8 showing the highest activity in this cell line (not shown). HERV-H19 was very active in HeLa cells. The HERV-HCM1 LTR exhibited the highest promoter activity in all cell lines and was especially active in lung fibroblasts (LC5) (Fig. 3).

3.4 Construction of HERV hybrid vectors and monitoring of the activities of HERV promoters in these vectors

The functionality of human endogenous retroviral LTR sequences in retroviral vectors was tested in two different promoter conversion vectors (ProCon). For this purpose, hybrid HERV/MLV vectors were constructed using two vectors

pLESN-MMTV (Fig. 7) and pLX-MMTV (Fig. 8) on the basis of MLV. These vectors include the EGFP gene as a reporter gene which is expressed from the 5' LTR (in varying amounts depending whether measured before or after the promoter conversion) as well as a neomycin gene which is expressed from an SV40 promoter. Moreover, vector pLX-MMTV contains a prokaryotic origin of replication enabling recloning of the provirus for further molecular characterizations.

To construct the HERV hybrid vectors, in each case the MMTV LTR was replaced by the HERV-HCM1 LTR (Fig. 7). For this purpose, the LTR was amplified first by means of PCR from the vector pBL-HERV-H using specific primers which contained additional sequences for the restriction enzymes MluI and SacII. Then, these fragments were inserted into the vectors having their 3' U3 deleted. After transfection into the packaging cell line, the EGFP reporter gene is first expressed from the MLV promoter (Fig. 9a). After infection of the target cells and successful promoter conversion by reverse transcription in the target cells, the reporter gene is present under the transcriptional control of the HERV LTR.

The HERV hybrid vector constructs pLESN-HERV-H (Fig. 7) and pLX-HERV-H (Fig. 8) as well as the parent vectors pLESN-MMTV and pLX-MMTV were transfected into the amphotrophic packaging cell line PA317. Afterwards, the resulting retroviral vector particles were used for the infection of cell lines CrfK and LC5.

The infected cell lines were cloned and the selected cellular clones were examined for the presence of vector constructs and for the occurrence of promoter conversion. For this purpose, chromosomal DNA was prepared from infected and

uninfected cells and analyzed by means of PCR. The primers were selected from the MLV U3 (P5) and R (P2) region as well as the HERV-H region (P1) and used for the PCR in combination with a primer for the EGFP region (Fig. 9a). The PCR products were hybridized with HERV-H-specific probes (Fig. 9b). After amplification with the primers P1 and P3, the DNA infected by pLX HERV-H particles yielded a PCR product of 1.1 kb which hybridized to the HERV-H probe. Amplification using MLV U3-specific primers (P2/P3) with DNA of cells infected with pLX and pLX HERV-H gave PCR products having a size of about 900 bp which showed not hybridization to the HERV-H probe. No PCR product hybridizing to the HERV-H probe was obtained from the amplification using MLV R primers (P5/P3). These results show that promoter conversion has occurred and that the MLV promoter of the 5' LTR had been replaced by the HERV promoter.

After integration into the target cell DNA, the HERV LTR promoter activity in the retroviral vectors was determined by FACS analyses via the measurement of the EGFP fluorescence (Fig. 10). For this purpose, the activity of the starting vector pLX-MMTV was compared with that of the HERV vector pLX-HERV-H (H6) prior to and after induction with dexamethasone. The vector containing the MMTV LTR may be activated by dexamethasone. The vector containing the HERV LTR is not activated by dexamethasone, however, its activity is by a factor of 10 higher as compared to the dexamethasone-stimulated MMTV hybrid vector.

3.5 Effect of regulatory elements in the R and U5 regions on the promoter activity of HERV sequences

In order to examine which sequence region is required for a functional HERV promoter, the effect of additional LTR sequences localized outside of the U3 region in the LTR was investigated in several examples. For this purpose, the activity in the luciferase assay of the U3 region of 7 HERV-K LTRs (HERV-K-T47D, L5, L50, L8, L9, L48, and L20/49) was compared to the activity of the corresponding U3-R fragments. It was surprisingly found that the different R regions are able to affect the promoter in the U3 region in a very different manner. In the group 1 LTRs (L5, L50, L8, L9) the presence of R sequences resulted in a marked increase in promoter activity in all cell lines tested (Fig. 4a). In contrast, in the group 2 LTRs (L20/L49) the HERV promoter activity is reduced by the R region (Fig. 4b). The HERV-K-T47D promoter (Fig. 5) and the L48 promoter (not shown) are substantially unaffected by the respective R sequences. Interestingly, in the case of the HERV-K-T47D LTR sequence regions localized downstream of the U3-R region and comprising the U5 region as well as the 3' non-translated region and the start of the *gag* gene have a clearly activating effect (Fig. 5).

A sequence analysis of the different R regions tested revealed that group 1 LTRs have a binding site for transcription factor SP1 in the R region which is missing from the R region in group 2 LTRs (Fig. 6). In contrast, the group 2 R region contains a potential binding site for factor TFS3 which acts as a repressor of transcription. This shows that the activity of HERV promoters may be modified by insertion of additional regulatory elements such as transcription factor binding sites, enhancer sequences, or negative-regulatory elements.

References

Anderssen, S., Sjottem, E., Svineng, G., Johansen, T. (1997). Comparative analyses of LTRs of the ERV-H family of primate-specific retrovirus-like elements isolated from marmoset, african green monkey, and man. Virology **234**. 14-30

Di Christofano, A., Strazzullo, M., Longo, L., La Mantia, G. (1995). Characterization and genomic mapping of the ZNF80 locus: expression of this zinc-finger gene is driven by a solitary LTR of ERV9 endogenous retroviral family. Nucl. Acid. Res. **23**. 2823-2830

Donehower L. A., Bohannon, R. C., Ford, R. A. (1990). The use of primers from highly conserved *pol* regions to identify uncharacterized retroviruses by the polymerase chain reaction. J. Virol. Methods **28**. 33-46

Emmerman, M., Temin, M. H. (1986). Comparision of promotor suppression in avian and murine retrovirus vectors. Nucl. Acid. Res. **14**. 9381-9396

Feuchter, A., Mager, D. (1990). Functional heterogeneity of a large family of human LTR-like promoters and enhancers. Nucl. Acid. Res. **18**. 1261-1270

Feuchter-Murthy, A. E., Freeman, J. D., Mager, D. L. (1993). Splicing of a human endogenous retrovirus to a novel phospholipase A2 related gene. Nucl. Acid. Res. **21**. 135-143

Goodchild N. L., Wilkinson, D. A., Mager, D. L. (1992). A human endogenous long terminal repeat provides a polyadenylation signal to a novel, alternatively spliced transcript in normal placenta. Gene, **121**. 287-294

Herrmann, M., Kalden, J. R. (1994). PCR and reverse dot hybridisation for the detection of endogenous retroviral transcripts. J. Virol. Methods **46**. 333-348

Hoppe-Seyler, F., Butz, K., zur Hausen, H. (1991). Repression of the Human Papillomavirus Type 18 Enhancer by the Cellular Transcription Factor Oct-1. J. Virol. **65**. 5613-5618

Kato-N., Shimotohno, K., VanLeeuwen, D., Cohen, M. (1990). Human proviral mRNAs downregulated in choriocarcinoma encodes zinc finger protein related to Kruppel. Mol. Cell. Biol. **10**. 4401-4405

Komurian-Pradel, F., G. Paranhos-Baccala, F. Bedin, A. Ounanian-Paraz, M. Sodoyer, C. Ott, A. Rajoharison, E. Garcia, F. Mallet, B. Mandrand, and H. Perron, 1999. Molecular cloning and characterization of MSRV-related sequences associated with retrovirus-like particles. Virology 260: 1-9.

Leib-Mösch, C., Haltmeier, M., Werner, T., Geigl, E.-M., Brack-Werner, R., Francke, U., Erfle, V., Hehlmann, R. (1993). Genomic distribution and transcription of solitary HERV-K LTRs. Genomics **18**. 261-269

Mager, D. L. (1989). Polyadenylation Function and Sequence Variability of the Long Terminal Repeats of Human Endogenous Retrovirus-like Family RTVL-H. Virology **173**. 591-599

Mann, R., and D. Baltimore. 1985, Varying the position of a retrovirus packaging sequence results in the encapsidation of both unspliced and spliced RNAs. J. Virol. 54:401-407.

Mold, D. E., Wu, T., Askin, F., Huang, R. C. (1997). Four classes of HERV-K long terminal repeats and their relative promoter strengths for transcription. Biomed. Science **4**. 78-82

Perbal, B., 1984. A Practical Guide to Molecular Cloning. John Wiley & Sons.

Price, J., Turner, D., Cepko, C. (1987). Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. Proc. Natl. Acad. Sci. **89**. 9237-9241

Rein, A., 1994, Retroviral RNA packaging: a review. Arch. Virol. Suppl. 9:513-22.

Saller, R. M., Öztürk, F., Salmons, B., Günzburg, W. (1998). Construction and characterization of a hybrid mouse mammary tumor virus/murine leukemia virus-based retroviral vector. J. Virol. **2**. 1699-1703

Salmons, B., Günzburg, W. H. (1993). Targeting of retroviral vectors for gene therapy. Human Gene Therapy **4**. 129-141

Sambrook, J., E. F. Fritsch, and T. Manatis. 1989. Molecular Cloning. Cold Spring Harbor Laboratory Press, New York, USA.

Schulte, A. M., Lai, S., Kurtz, A., Czubayko, F., Riegel, A. T. (1996). Human trophoblast and choriocarcinoma expression of the growth factor pleiotrophin attributable to germ-line insertion of an endogenous retrovirus. Proc. Natl. Acad. Sci. **93**. 14759-14764

Seifarth, W., Baust, C., Murr, A., Skladny, H., Krieg-Schneider, F., Blusch, J., Werner, T., Hehlmann, R., Leib-Mösch, C., (1998). Proviral Structure, chromosomal location and expression of HERV-K-T47D, a novel human endogenous retrovirus derived from T47-D particles. J. Virol. **72**. *In press*

Shih, A., Misra, R., Rush, M. G. (1989). Detection of multiple, novel reverse transcriptase coding sequences in human nucleic acids: relation to primate retroviruses. J. Virol. **63**. 64-75

Sorge, J., Hughes, S. H. (1982). Polypurine tract adjacent to the U_3 region of the Rous sarcoma virus genome provides a *cis*-acting function. J. Virol. **43**. 482-488

Ting, C.-N., Rosenberg, M. P., Snow, C. M., Samuelson, L. C., Meisler, M. H. (1992). Endogenous retroviral sequences are required for tissue-specific expression of a human salivary amylase gene. Genes Dev. **6**. 1457-1465

Wilkinson, D. A., Mager, D. L., Leong, J.-A. (1994). Endogenous human retroviruses. In "The retroviridae" Vol. **3** (J. Levy, ed.), pp. 465-535, Plenum Press, NY.

Patience, C., Wilkinson, D. A., Weiss, R. A. (1997). Our retroviral heritage. Trends Genet. 13, 116-120.

Patience, C., Takeuchi, Y., Cosset, F.-L., Weiss, R. A. (1998). Packaging of endogenous retroviral sequences in retroviral vectors produced by murine and human packaging cells. J. Virol. 72, 2671-2676.